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Characterization of CTLA-4 Structure and Expression on Human T Cells[1]

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Abstract. CTLA-4 is an adhesion receptor expressed on activated T cells. The amino acid sequence of CTLA-4 is related to CD28, and although the function of CTLA-4 remains unknown, it shares several features with CD28, including a common counter-receptor, B7, that is present on Ag-presenting cells. In a recent study we found that CD28 and CTLA-4 were coexpressed at the mRNA level on activated T cells but that only CD28 was expressed on resting T cells. Here we show that within the T cell population, CTLA-4 expression is restricted to the subset of T cells that also express cell surface CD28. CTLA-4 mRNA expression can be induced on quiescent T cells via phorbol ester-mediated activation of protein kinase C but not with calcium ionophore treatment alone. Phorbol ester-induced expression of CTLA-4 mRNA could be enhanced with calcium ionophore treatment, and treatment of cells in this manner resulted in a reciprocal decrease in expression of CD28 mRNA. Ligation of CD28 with monoclonal antibody also resulted in the specific and rapid induction of CTLA-4 mRNA. To study the expression of CTLA-4 at the protein level, a rabbit antiserum against a recombinant protein derived from CTLA-4 cDNA was generated. When activated T cells were labeled with [^{35}S]methionine, the rabbit antiserum precipitated a 41- to 43-kDa protein from whole cell lysates. Similar results were found when detergent-soluble lysates from ^{125}I surface-labeled resting and activated T cells were analyzed by SDS-PAGE. Surprisingly, under the conditions tested, CTLA-4 migrated primarily as a monomer at the cell surface, and could not be shown to exist as a disulfide-bonded homodimer or as a heterodimer consisting of CTLA-4 and CD28. These results suggest that B7 can bind to T cells via distinct receptor complexes consisting of either CD28 or CTLA-4, and that these complexes may potentially mediate distinct biologic functions. Further, the present results suggest that noncovalent interactions might mediate association of CTLA-4 and/or CD28 at the cell surface. *Journal of Immunology,* 1993, 151: 3489.

Normal T cells or T cell clones do not proliferate in response to Ag unless additional signals are provided by Ag presenting cells (APC) (1). In most cases, the costimulatory signal provided by the APC requires cell contact, although in some instances soluble factors have been shown to provide an efficient costimulatory signal (2). The B7 antigen is one molecule on APC that is capable of providing costimulatory signals to T cells via cell contact (3–5). The B7 receptor is a 44 to 54 kDa Ag (6, 7) that is expressed on all APC of lymphoid origin examined to date (6, 8, 9). Recent studies have shown that B7 is the counter-receptor for both the CD28 and CTLA-4

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receptors expressed on T cells (10, 11).

Within the Ig gene superfamily, CD28 and CTLA-4 are closely related adhesion molecules that are members of the subfamily that contain single V-like domains (12, 13). The genomic colocalization of CTLA-4 and CD28 within 50 kb of each other, on band q33 of human chromosome 2 (14 and T. Lindsten, unpublished data), and on mouse chromosome 1 band C (15), suggests that the receptors arose by gene duplication, and that this event occurred before speciation (16, 17).

In both the murine and human immune systems, CD28 seems to have an important role in the delivery of costimulatory signals (reviewed in Reference 18). Experiments using mAb as a ligand of the CD28 receptor have shown that CD28 can regulate lymphokine expression by both transcriptional and post-transcriptional mechanisms (19, 20). Anti-CD28 mAb prevents the development of clonal anergy in mouse T cell clones (21). Several laboratories have shown that the interaction of T cells with B7 expressed on lymphoid and nonlymphoid cells is capable of regulating T cell lymphokine production (3, 4, 22) and is able to induce T cell cytotoxicity (23). Recent studies in vivo suggest that the interaction of B7 with ligand is necessary for efficient cell-mediated rejection of allografts (24–26).

CTLA-4 was originally identified as the fourth cDNA during a search for genes that are specifically expressed in cytotoxic T cells, and hence the name "cytolytic T lymphocyte associated antigen," CTLA-4 (13). The genes for both human and mouse CTLA-4 share a similar exon and intron structure with each other and with human and mouse CD28 (17, 27), and exhibit extensive homology at the nucleotide level. The extended interspecies conservation of CTLA-4 is seen in the high degree of overall homology between the human and mouse proteins (>70%), and in the complete identity of the cytoplasmic domains (17). In human T cells, CD28 and CTLA-4 mRNA are coexpressed on activated $CD4^+$ and $CD8^+$ cells, and in nearly all T cell clones (28) In the mouse, CTLA-4 mRNA is not expressed in resting T cells, and is expressed in activated T cells, including $CD8^+$ CTL (13) and in $CD4^+$ T cell clones of both Th1 and Th2 subtypes (28). In contrast, CD28 is expressed on the surface of most resting T cells and thymocytes predominantly as a disulfide-bonded homodimer (29–32). Despite the similarities between the CD28 and CTLA-4 genes and their expression in T lymphocytes, a role for CTLA-4 in T cell activation has not been established. In the present study we report the requirements for CTLA-4 gene expression at the mRNA and protein levels. In T cells, CTLA-4 mRNA expression was found to be restricted to the $CD28^+$ subset. The binding of human CD28 mAb to T cells was found to increase CTLA-4 mRNA expression. An antiserum that specifically recognizes CTLA-4 immunoprecipitated a 41- to 43-kDa protein from the cell surface of activated T cells. Surprisingly, on SDS-PAGE analysis CTLA-4 isolated from the surface of cells migrated primarily as a monomer, and therefore, contrary to expectations, is not present as a disulfide-bonded dimer.

Materials and Methods

Antibodies

CD28 mAb 9.3 and CD3 mAb G19.4 were produced and purified as previously described (33). In cell culture experiments mAb G19.4 was cross-linked to the surface of plastic dishes at a concentration of 1 μg/ml, as previously described (34, 35). mAb 9.3 was used at final concentration of 1 μg/ml in cell cultures. Rabbit antiserum 1438 against the extracellular domain of hCTLA-4 was generated as follows: human CTLA-4 plasmid pRIV2/7914 was digested with *Pml*I and *Bam*HI and a ~385-bp fragment encoding most of the extracellular domain of CTLA-4 was purified by electrophoresis in low temperature gelling agarose. The fragment was ligated into the *Eco*RV/*Bam*HI sites of the expression vector pRDC2.2. The ligated product was transformed into *Escherichia coli* JM105, selected by ampicillin resistance, and the recombinant plasmids verified by restriction enzyme digestion. Expression vector pRDC2.2 directs the constitutive expression of fusion proteins in which the gene of interest is fused to the first ~25 amino acids of β-glucuronidase. Plasmid DNA containing the extracellular domain of CTLA-4 (pCTLA4–2) was transformed into *E. coli* expression strain RGN715.

The CTLA-4 expression strain (RGN715/pCTLA4–2) was grown in 2× Luria Broth overnight at 37°C with constant shaking. The cells were collected by centrifugation, suspended in lysis buffer (50 mM Tris, pH 7.6, 5 mM EDTA, 0.5% Triton X-100), and lysozyme (2 mg/g cell paste) was added. The cells were incubated for 1 h at 37°C, $MgSO_4$ was added to a final concentration of 15 mM, DNase was added to 500 U/100 g cell paste, and the cells were incubated for an additional 15 min. The cells were then homogenized and lysed using repeated combinations of polytron/sonication. The inclusion bodies containing recombinant human CTLA-4 were collected by centrifugation and washed twice with lysis buffer.

The inclusion bodies containing recombinant CTLA-4 at >50% purity were solubilized in extraction buffer (50 mM Tris, pH 7.6, 8 M urea, 5 mM EDTA, 10 mM dithiothreitol, 0.5% Triton X-100), buffer exchanged into 25 mM Tris, pH 7.6, 8 M urea, 5 mM EDTA, 10 mM β-ME and chromatographed on a DEAE column. Under these conditions, the CTLA-4 appears in the column flowthrough and most contaminating proteins were retained by the DEAE. The flowthrough fraction containing soluble human CTLA-4

(hsCTLA-4)[4] was then chromatographed on a Sephadex S-200 (Pharmacia, Piscataway, NJ) sizing column, and the fractions containing recombinant hsCTLA-4 (>90% pure) were pooled.

Polyclonal antisera to the recombinant hsCTLA-4 was prepared by immunizing rabbits with 250 μg hsCTLA-4 in complete Freund's adjuvant and boosting at 2-wk intervals with 125 μg in incomplete Freund's adjuvant. Rabbits were bled 2 wk after the final boost, and the titer of anti-CTLA-4 antibodies was determined by ELISA. Rabbit 1438 yielded a high titer anti-CTLA-4 antiserum. ES5.2D8 is an IgG2b monoclonal antibody that binds to activated T cells and was used as a control in some experiments. KT3 mAb was a gift from G. Walter (36).

Cells

Human $CD28^+$ T cells were purified by a previously described negative selection method (35). To obtain $CD28^-$ T cells, negative selection using immunobeads was preformed as previously described (37), taking advantage of the reciprocal expression of the cell surface Ag CD11b and CD28 on T cells. Cells were cultured at 2×10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES. Cells were stimulated with cross-linked anti-CD3 mAb G19–4 with or without anti-CD28 mAb 9.3 (34, 35). PMA or PDBU (Sigma, St. Louis, MO) was used at a final concentration of 10 or 100 ng/ml, and ionomycin (Calbiochem, La Jolla, CA) at 0.4 μg/ml.

Northern blot analysis

Cells were harvested after stimulation and total RNA was extracted and equalized for rRNA, as previously described (35, 38). Equalized RNA samples were separated on 1% agarose/formaldehyde gels and transferred to nitrocellulose. The following DNA probes, labeled by nick translation, were used: a 1.5-kb CD28 cDNA (12) and a 1.4-kb HLA-B7 cDNA (39). A CTLA-4 cDNA clone was obtained by the polymerase chain reaction (PCR) using RNA from $CD28^+$ T cells stimulated with anti-CD3 plus anti-CD28 for 24 h. The CTLA-4 cDNA sequence has been described previously (17). First strand cDNA synthesis of the predicted coding region was performed (27) utilizing a 3′ oligonucleotide primer (GGGC GGC CGC CAT AAT GGT TTC [TCA] ATT GAT GGG AAT) which spans the stop codon (brackets) in exon 4. PCR was then performed using the same 3′ oligonucleotide primer and 5′ primer (GG GGA AGC TTA GCC [ATG] GCT TGC CTT GGA TTT CAG CGG CA) which spans the predicted initiation codon (brackets). PCR was performed for 30 cycles (94°C for 1.5′, 56°C for 1′, 72°C for 4′, followed by 72°C × 10′ final extension step). PCR products were subsequently digested with *HindIII* and NotI (to cleave restriction enzyme sites contained in the oligonucleotide primers) and cloned into Bluescript SK plasmid vector (Stratagene, San Diego, CA). The veracity of the CTLA-4 cDNA clones was confirmed by DNA sequencing using the Sequenase kit according to supplier's protocol (U.S. Biochemical Corp., Cleveland, OH).

In vitro transcription and translation of CTLA-4 protein

To facilitate identification of the CTLA-4 protein, a genetic chimera was produced by fusing the epitope recognized by the KT3 mAB to the 3′ end of the CTLA-4 coding region (40). Using the CTLA-4 cDNA clones derived above as DNA templates, PCR was performed using the same 5′ oligonucleotide primer and a 3′ oligonucleotide primer (GGC GGC CGC [TCA] TGT TTC TGG TTC TGG TGG TGG TGT **ATT GAT GGG AAT AAA**) which overlaps the last 5 codons of the CTLA-4 coding region (bold) and the stop codon (brackets) and contains the epitope from the 3′ end of the SV40 large T antigen recognized by KT3 (underlined). PCR, subcloning, and sequence verification were performed as above. In vitro transcription and translation were then performed according to manufacturer's protocol (Promega Gemini System II Ribo probe p2020 and Promega Wheat Germ Lysate, Promega, Madison, WI) using the fusion construct. Translation was performed in the presence of 10 mM final concentration of sodium acetate and 0.8 mCi/ml [^{35}S]methionine (in vitro translation grade, specific activity >1000 Ci/mmol, ICN, Irvine CA). Immunoprecipitations were performed on 20% of the total translation mix in a final volume of 100 μl, as outlined below.

Metabolic labeling and immunoprecipitation

Purified $CD28^+$ T cells were washed twice in methionine-free, cysteine-free DMEM (Gibco-BRL, Grand Island, NY). The cells were then resuspended at 1×10^6/ml in 9 parts methionine-free, cysteine-free DMEM supplemented with 10% dialyzed fetal calf serum and 1 part RPMI supplemented with 10% fetal calf serum. To the cell suspension were added 200 μCi/ml [^{35}S]methionine (Translabel, ICN), PMA at a final concentration of 10 ng/ml, and ionomycin at 0.4 μg/ml. The cells were activated for 6 to 36 h to induce CTLA-4 expression. After biosynthetic labeling and cellular stimulation, the cells were solubilized in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 0.1 mM EGTA, 1 mM EDTA, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.01 mM *N*-tosyl-L-lysine chloromethyl ketone (TLCK), 0.4 mM phenylmethanesulfonyl fluoride (PMSF),

[4] Abbreviations used in this paper: hsCTLA-4, recombinant soluble human CTLA-4; PCR, polymerase chain reaction.

and 1.2 μM aprotinin) at 10×10^6 cells/ml. Immunoprecipitations were performed with an aliquot of lysate (250 μl) representing 2.5×10^6 cell equivalents. After preclearing the extracts with a 2% final solution of Pansorbin (Calbiochem, La Jolla, CA), 1 μl of the 1438 antiserum was added, followed by 2 h incubation at 4°C. In immunoprecipitations with the in vitro translated CTLA-4/SV40 large T antigen fusion protein, 25 μl or 50 μl of culture supernatant from the KT3 hybridoma, or 10 μl of ascites from the ES5.2D8 hybridoma was used. 50 μl of a 3% protein A Sepharose (Pharmacia) solution was then added, and the incubation was continued on a rocking platform at 4° for 2 h. The immunoprecipitates were then washed in 10 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, and 0.1% SDS, dissolved in SDS sample buffer in the presence of β-ME, boiled, and loaded on a 9.5% SDS-polyacrylamide gel. After electrophoresis, the gels were fixed in 40% methanol, 10% glacial acetic acid, enhanced with Amplify (Amersham, Arlington Heights, IL), dried, and exposed to x-ray film. In blocking experiments, 1 μl of the 1438 antiserum was diluted in 10 μl of phosphate-buffered saline and incubated with 10 μl of hsCTLA-4 at 0.9 mg/ml for 2 h at room temperature.

Cell surface labeling and immunoprecipitation

Purified T cell subsets were cultured as indicated, washed, and lactoperoxidase-catalyzed cell surface iodination performed as described (41). Cells were lysed in ice-cold buffer (50 mM Tris, pH 7.6, 0.5% Triton X-100 or 30 mM octylglucoside, 150 mM NaCl, 5 mM EDTA, 10 μg/ml aprotinin and leupeptin, 25 μg/ml *p*-nitrophenyl-guanidinobenzoate). 10 mM iodoacetamide was included in the lysis buffer to alkylate free sulfhydryl groups. Detergent soluble supernatants were precleared with preimmune rabbit serum and protein A agarose, followed by immunoprecipitation with 1438 serum or mAb 9.3 for 4 h. Protein A Sepharose CL-4B (Pharmacia) was used to precipitate the immune complexes. The beads were washed, bound proteins were eluted by boiling in sample buffer, followed by SDS-PAGE on 8.5% to 12.5% gels, and the results were assessed by phosphoimagery (Phosphorimager, Molecular Dynamics, Sunnyvale, CA).

Results

CTLA-4 gene expression is limited to the CD28$^+$ subpopulation of T cells

CD28 is expressed on the surface of thymocytes, a major subset of resting and activated T cells, and on plasma cells. In contrast, available studies indicate that expression of CTLA-4 mRNA is more limited and is restricted to activated T cells (17), HTLV-1 transformed T cell lines (28), and possibly activated murine splenic B cells (13). In agreement with previous studies, we did not detect CTLA-4 mRNA in purified resting peripheral blood T cells (Fig. 1).

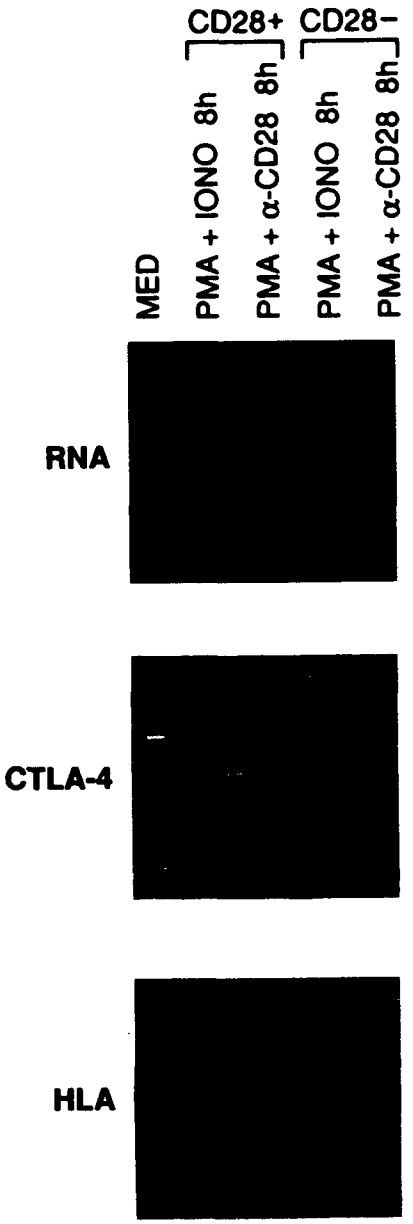

FIGURE 1. Expression of CTLA-4 mRNA in T cell subpopulations. Resting T cells were cultured in medium (*MED*) or fractionated into the CD28$^-$ and CD28$^+$ subsets using magnetic immunobead negative selection. The cells (2×10^6/ml) were cultured in the presence of PMA (3 ng/ml) plus ionomycin (0.4 μg/ml) (*PMA + IONO*), or PMA plus soluble anti-CD28 mAb 9.3 (1 μg/ml) for 8 h (*8 h*). RNA was isolated and equalized for rRNA, and the equalization confirmed by ethidium bromide staining (*upper panel*). Northern blots were prepared and the filters were hybridized sequentially with cDNA probes specific for CTLA-4 and HLA class I mRNA.

Previous studies have shown that CTLA-4 mRNA is expressed in activated CD4$^+$ and CD8$^+$ T cells (28). CD28 defines a subpopulation of T cells that includes ~95% of CD4$^+$ cells and about 50% of CD8$^+$ cells. To examine expression of CTLA-4 mRNA across this subset of T cells, peripheral blood T cells were separated into >95% pure

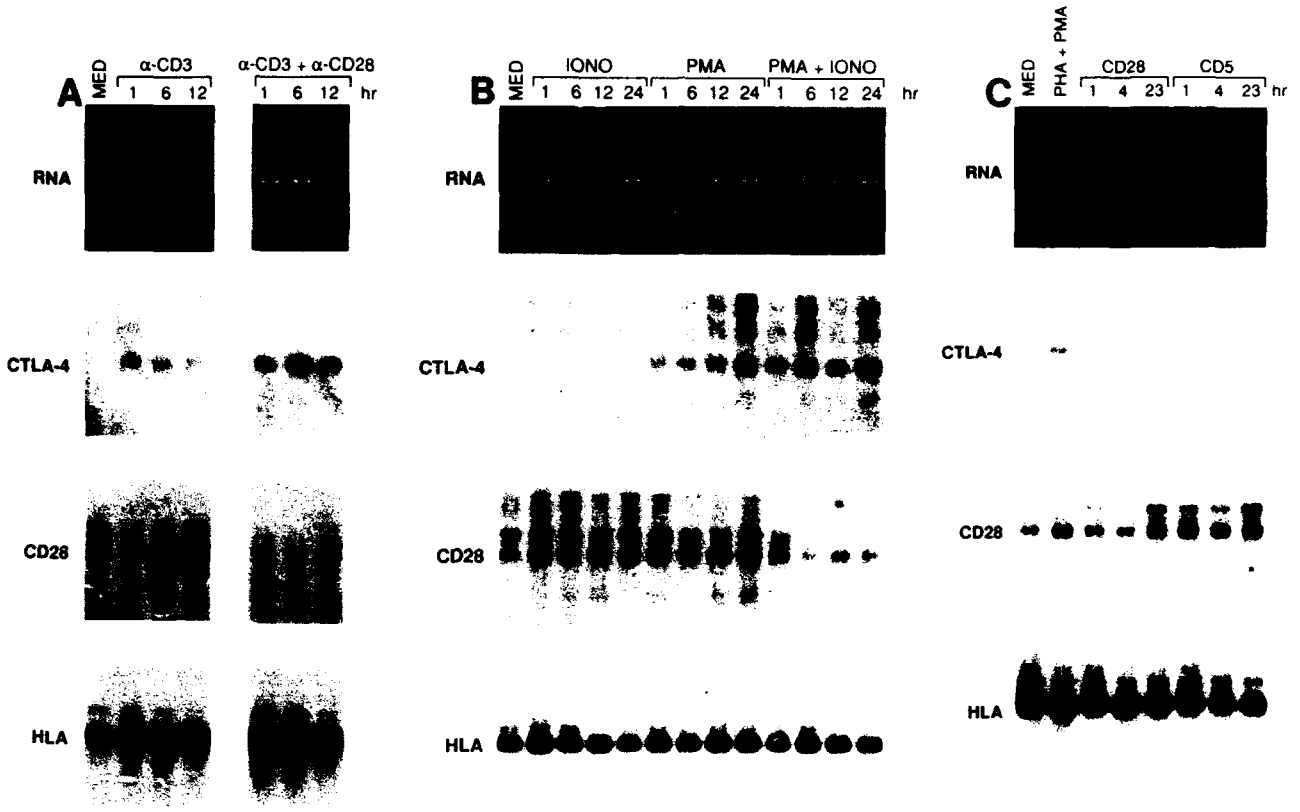

FIGURE 2. Requirements for induction of CTLA-4 gene expression. Peripheral blood $CD28^+$ T cells (2×10^6/ml) were cultured in the presence of (*A*) medium (*MED*), plastic immobilized anti-CD3 mAb G19–4, or anti-CD3 mAb plus soluble anti-CD28 mAb 9.3 (0.5 μg/ml) for 1, 6, or 12 h; (*B*) medium (*MED*), ionomycin (*IONO*) (0.4 μg/ml), PMA (3 ng/ml), or PMA plus ionomycin (*PMA + IONO*) for 1, 6, 12, or 24 h; or (*C*) medium (*MED*), PHA 20 μg/ml plus PMA 10 ng/ml for 23 h, or biotinylated anti-CD28 mAb 9.3 (*CD28*) or biotinylated anti-CD5 mAb 10.2 (*CD5*) plus avidin for 1, 4, or 23 h. In all experiments, the cells were collected after stimulation, lysed, and Northern blots prepared as described in Figure 1.

$CD28^+$ and $CD28^-$ populations (37). The cells were stimulated for 8 h with PMA and ionomycin, or with PMA and anti-CD28 mAb, and the expression of CTLA-4 was determined by Northern blot analysis. CTLA-4 expression was limited to the $CD28^+$ population of T cells (Fig. 1). Thus, these results confirm that CTLA-4 mRNA is only expressed in activated T cells. Further, a subpopulation of T cells has been identified that does not express CTLA-4 mRNA. These results also imply that all cells that express CTLA-4 mRNA coexpress CD28.

Stimulation requirements for CTLA-4 gene expression

To characterize the signals required for induction of CTLA-4 gene expression, purified $CD28^+$ T cells were subjected to a variety of stimuli. As expected from previous studies (17, 28), T cells expressed CTLA-4 mRNA after stimulation with the potent and relatively nonspecific mitogenic combination of PHA and PMA (Fig. 2*C*). CTLA-4 gene expression is also rapidly induced by cross-linking of the T cell receptor/CD3 complex with an anti-CD3 monoclonal antibody (Fig. 2*A*). CTLA-4 mRNA is present within 1 h after anti-CD3 stimulation and peaks at 6 h. CD28 receptor costimulation leads to an augmentation in CTLA-4 gene expression compared with that induced by anti-CD3 treatment alone (Fig. 2*A*). To further dissect the signals necessary to induce CTLA-4 gene expression, T cells were cultured in the presence of the pharmacologic agents ionomycin and PMA (Fig. 2*B*). Mobilization of intracellular calcium following stimulation with ionomycin alone did not induce CTLA-4 gene expression. In contrast, protein kinase C activation by PMA alone was sufficient to induce CTLA-4, with expression continuing to increase throughout the 24-h experiment. However, the combination of PMA and ionomycin stimulation had a synergistic effect, and resulted in earlier peak levels of CTLA-4 mRNA expression. A biphasic course of CTLA-4 mRNA expression was found after PMA plus ionomycin stimulation, with early and late peaks of mRNA levels. It is not yet known if this is a consistent finding, because further experiments to test this observation have not been performed. These early and late CTLA-4 peaks were specific, because the level of HLA class I mRNA remained relatively constant throughout the experiment.

The primary signal transduction pathways activated by

the CD28 receptor remain to be determined, but are known to involve calcium mobilization and protein tyrosine phosphorylation that is distinct from the T cell receptor (42, 43). Interestingly, cross-linking of the CD28 surface structure also leads to the rapid (≤1 h) induction of CTLA-4 mRNA as shown in Figure 2*C*. Induction of CTLA-4 expression by CD28 was somewhat specific, because cross-linking of CD5 resulted in transient, low-level CTLA-4 mRNA expression at the 1-h time point. Inhibition of anti-CD28-induced tyrosine phosphorylation with herbimycin C prevents the induction of CTLA-4 gene expression (data not shown). It should be noted that CD28 mRNA expression is present in resting cells, and in agreement with previous studies (44), is further induced by PMA or anti-CD3 stimulation. Interestingly, CD28 and CTLA-4 mRNA appear to be reciprocally expressed under some conditions. Combined treatment of T cells with PMA and ionomycin leads to the induction of CTLA-4 mRNA, and to a concomitant decrease in CD28 mRNA (Fig. 2*B*). The decrease in CD28 mRNA expression was most marked at 6 h after stimulation, and represented three- to fourfold decrease compared to baseline levels, as estimated by densitometric analysis of the autoradiogram (data not shown). Thus, CTLA-4 gene expression is induced after protein kinase C activation via PMA stimulation, and by cross-linking of the TCR/CD3 complex. CD28 costimulation, under conditions leading to substantial augmentation of T cell lymphokine production, also augments CTLA-4 mRNA expression. In addition, cross-linking of CD28 alone is sufficient to induce CTLA-4 mRNA accumulation.

CTLA-4 mRNA is not expressed nor induced in the Jurkat T cell line

We compared the expression of CTLA-4 in normal T cells to the Jurkat leukemia T cell line. Similar to normal, resting $CD28^+$ T cells, CTLA-4 is not expressed in unstimulated Jurkat T cells, while CD28 was constitutively expressed in both cell types, although at substantially higher levels in Jurkat cells (not shown). However, unlike normal T cells, treatment of Jurkat cells with PHA plus PMA did not induce CTLA-4 expression. In addition, treatment of Jurkat cells with PMA plus ionomycin or PMA plus ionomycin plus anti-CD28, conditions that induce IL-2 gene expression in Jurkat, did not induce CTLA-4 gene expression. Thus, induction of CTLA-4 expression is not required for induction of IL-2 synthesis, at least in transformed cell lines.

Characterization of rabbit anti-hsCTLA-4 antiserum

Previous studies have not identified or characterized CTLA-4 protein expression. To determine the structure of the CTLA-4 protein on the surface of the T cell and its possible relationship to CD28, rabbits were immunized with a bacterially derived fusion protein containing the extracellular domain of CTLA-4 (hsCTLA-4) as described in *Materials and Methods*. To establish specificity of the antiserum, a construct was made containing the full-length CTLA-4 cDNA to which eight amino acids from the C-terminus of the SV40 large T Ag were added as an epitope tag to the C-terminus of CTLA-4. The fusion protein containing this epitope tag is recognized by the KT3 mAb (36, 40) and was produced by in vitro transcription and translation. As can be seen in Figure 3*A*, one antiserum, 1438, precipitates material that migrates at 25 and 17 kDa from the in vitro translated CTLA-4/SV40 large T Ag construct. The 25-kDa band is assumed to be full-length CTLA-4 fusion protein, because it corresponds well to the predicted migration of the construct (24.9 kDa), whereas the 17-kDa material likely represents partially transcribed

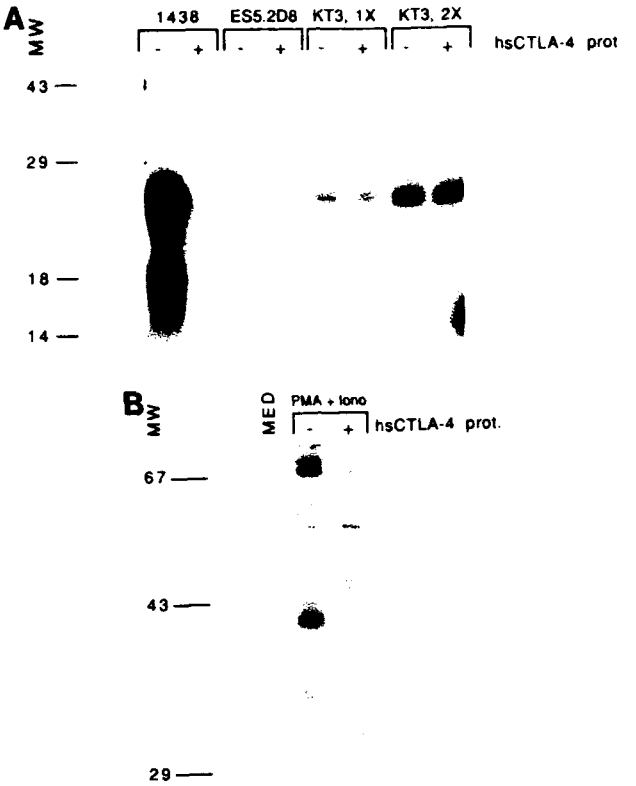

FIGURE 3. CTLA-4 protein expression in [^{35}S]methionine-labeled lysates from an in vitro translation mix or from activated T cells. *A*, In vitro-translated CTLA-4/SV40 large T antigen fusion protein was subjected to immunoprecipitation with rabbit anti-CTLA-4 antiserum 1438, KT3 mAb at two different concentrations (*1*× and *2*×), or control mAb ES5.2D8 (see *Materials and Methods* for details). The antiserum or mAb were preincubated in the presence (+) or absence (–) of hsCTLA-4 before immunoprecipitation of the wheat germ lysates. *B*, Rabbit anti-CTLA-4 antiserum 1438 precipitates a 41-kDa protein (←) from activated T cells. $CD28^+$ T cells were labeled with [^{35}S]methionine during a 12-h stimulation with PMA plus ionomycin, and whole cell lysates subjected to immunoprecipitation. Antiserum 1438 was preincubated in the presence (+) or absence (–) of hsCTLA-4.

material. No immunoprecipitation was observed if the antiserum 1438 was preincubated with the immunogen (Fig. 3*A*). KT3 also was found to immunoprecipitate a 25-kDa protein, which was not inhibitable with the immunogen hsCTLA-4 (Fig. 3*A*). On longer exposures, the 17-kDa protein seen in the immunoprecipitations with 1438 was also present in the KT3 immunoprecipitates (not shown). Further, the effect of KT3 was specific because a control mAb did not precipitate the in vitro translated CTLA-4/SV40 large T antigen protein (Fig. 3*A*).

CTLA-4 is expressed as a 41- to 43-kDa protein in lysates of ^{35}S-labeled activated T cells

With the specificity of the 1438 antiserum established, we set out to identify and characterize the structure of the CTLA-4 protein in purified CD28$^+$ T cells. T cells were cultured for 12 h in the presence of PMA plus ionomycin to induce CTLA-4 mRNA expression, and were labeled with [^{35}S]methionine, as described in *Materials and Methods*. Postnuclear cell lysates were prepared and immunoprecipitated with the 1438 antiserum (Fig. 3*B*). The 1438 antiserum immunoprecipitates multiple bands from activated but not resting T cells, including material from T cell blasts that migrates at 41 to 43 kDa under reducing conditions. In contrast to the in vitro translated protein which has an apparent molecular mass of 25 kDa, the predominant cellular form of CTLA-4 recognized by antiserum 1438 has a migration of 41 to 43 kDa. This range is presumably caused by a variable amount of glycosylation or other processing. Similar results were obtained when T cell blasts were prepared by stimulation with cross-linked anti-CD3+IL-2 (data not shown). Complete inhibition of the 41- to 43-kDa bands and partial inhibition of the 34 kDa and 70- to 75-kDa bands was observed when the 1438 antiserum was preincubated with hsCTLA-4 (Fig. 3*B*), suggesting that these bands represent CTLA-4 in various stages of assembly or degradation. Alternatively, some of the bands could represent molecules associated with CTLA-4, or cross-reactive material of unknown identity.

CTLA-4 is expressed as a 41- to 43-kDa monomer on the surface of activated T cells

To study surface expression of CTLA-4, purified CD28$^+$ T cells were stimulated under a variety of conditions, and surface-labeled with lactoperoxidase-catalyzed radioiodination (Fig. 4). Consistent with previous reports (29–32), a wide band that was centered at 44 kDa was immunoprecipitated by CD28 mAb 9.3 under reducing conditions from both resting and PMA plus ionomycin-activated T cells. Approximately 30-fold more CD28 was precipitated from activated T cells than from resting T cells (Fig. 4*A*). In contrast, when lysates from T cell blasts were subjected to immunoprecipitation with the 1438 antiserum, a 41- to 43-kDa band was observed that was more sharply defined than CD28. Minor bands were also observed at 35 kDa and 90 kDa, but were observed less consistently and represented less than 10% of precipitated counts. Prolonged exposure of the gels from resting T cells revealed that small amounts of material were precipitated by 1438 (Fig. 4*A*, *left panel*). This material also migrated at 41- to 43-kDa, and was more than 300-fold less intense than the amount found in immunoprecipitates from activated T cells.

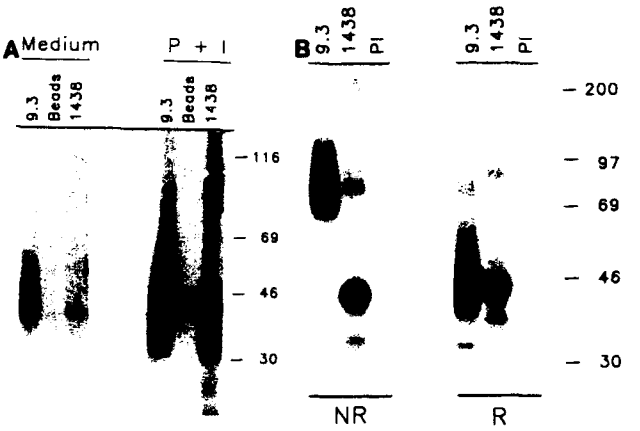

FIGURE 4. CTLA-4 is expressed as a 43-kDa monomer on the surface of activated T cells. CD28$^+$ peripheral blood T cells were isolated and surface labeled with ^{125}I by lactoperoxidase-catalyzed iodination. *A*, Cells were cultured in medium or PMA plus ionomycin (*P + I*) for 26 h, labeled, and membrane proteins solubilized in lysis buffer containing 30 mM octylglucoside. Immunoprecipitation of 10^6 cpm/sample was done with anti-CD28 mAb 9.3, anti-CTLA-4 antiserum 1438, or protein A beads alone (*Beads*), followed by 8.5% SDS-PAGE under reducing conditions. *B*, Cells were stimulated with PHA plus PMA for 24 h, labeled, and immunoprecipitations with mAb 9.3, rabbit antiserum 1438 or preimmune rabbit serum (*PI*) performed under reducing (*R*) or nonreducing (*NR*) conditions, followed by SDS-PAGE on an 8.5% gel.

CTLA-4 is not detected as a disulfide-bonded homo- or heterodimeric receptor

The predicted amino acid sequence of both human and mouse CTLA-4 and CD28 contains 5 cysteine residues in the extracellular domain (17). Four of these residues are expected to form the intrachain disulfide bonds required for the Ig-like V region, whereas a fifth membrane proximal cysteine exists. This unpaired cysteine is thought to be involved in the formation of a disulfide-bonded homodimer in the case of CD28, and has been predicted to function similarly in CTLA-4 (17). T cell blasts were prepared by stimulation with PHA plus PMA and were surface iodinated. When lysates from these cells were analyzed, 1438 again immunoprecipitated a band at 41 to 43 kDa under reducing conditions, while CD28 migrated as a more diffuse band (Fig. 4*B*). Surprisingly, material precipitated by

1438 also migrated at 41 to 43 kDa under nonreducing conditions, whereas, as expected, CD28 migrated as a broad smear centered at 90 kDa (Fig. 4*B*). The material precipitated by 1438 displayed somewhat less mobility under reducing than in nonreducing conditions, consistent with the presence of intrachain disulfide bonds. Preimmune serum from rabbit 1438 did not precipitate material from these lysates.

We considered that antiserum 1438 might recognize CD28, given the homology at the protein level. Although this was unlikely considering the scant amount of material precipitated by 1438 from CD28$^+$ resting T cells (Figs. 3*B* and 4*A*), immunoprecipitations were performed on Chinese hamster ovary cells transfected with CD28. The rabbit 1438 antiserum did not immunoprecipitate any protein from the CD28 transfectants, although anti-CD28 mAb 9.3 precipitated large amounts of CD28 (data not shown).

It was also possible that CTLA-4 might pair with CD28 and form a heterodimeric receptor, given the presence of an "unpaired" cysteine in CTLA-4 and CD28, and given that free subunits of CD28 can exist on the cell surface (29). This could potentially account for the small amount of material in the 90-kDa range that was immunoprecipitated with antiserum 1438. To test this possibility, T cell blasts were surface-labeled, and detergent lysates were prepared under nonreducing conditions. The lysates were precleared with anti-CD28 mAb 9.3 or anti-CTLA-4 serum 1438. The material precipitated by 1438 was not depleted in lysates precleared with anti-CD28, although CD28 was efficiently precleared (Fig. 5). Similarly, anti-CTLA-4 serum 1438 did not preclear the CD28 glycoprotein (Fig. 5).

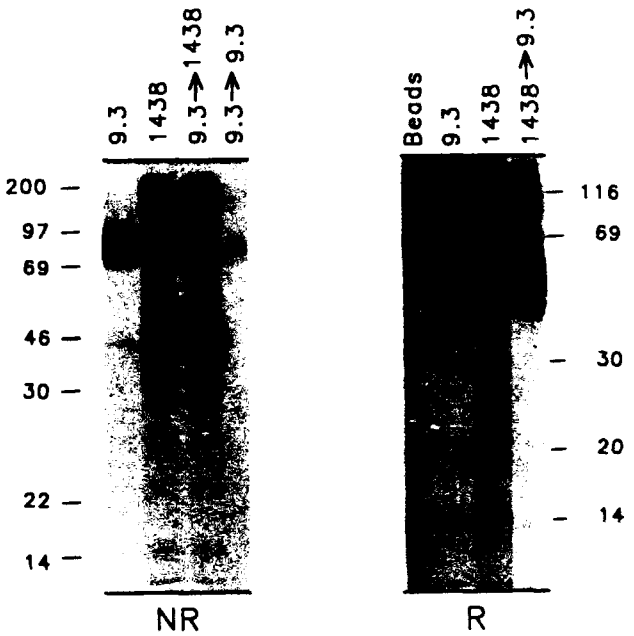

FIGURE 5. CTLA-4 and CD28 do not coprecipitate. CD28$^+$ peripheral blood T cells were stimulated in PHA plus PMA for 1 day (*left panel*) or for 4 days (*right panel*). After lysis and extraction in detergent buffer (0.5% Triton X-100), lysates were precleared with mAb 9.3 followed by a secondary immunoprecipitation with 1438 (9.3 → 1438) or 9.3 (9.3 → 9.3; *left panel*) or they were precleared with 1438 followed by immunoprecipitation with 9.3 (1438 → 9.3; *right panel*). Extracts were also immunoprecipitated with 9.3 and 1438 alone or with protein A beads (*Beads*) before SDS-PAGE on a 12.5% gel under reducing (*R*) or nonreducing (*NR*) conditions. The positions of molecular mass markers are indicated in kilodaltons.

Discussion

In the present study we have used T cells activated by a variety of stimuli to study CTLA-4 expression and structure. CTLA-4 mRNA expression was not detectable in quiescent T cells, although it was induced within 1 h after ligation of the TCR or CD28 receptor. The rapid expression of CTLA-4 mRNA was surprising because previous studies had not examined expression during the early phases of T cell activation. The significance of the early expression remains to be determined, but suggests that CTLA-4 protein is available on the surface of T cells to function in the G1 phase and in subsequent phases of the cell cycle.

Both protein kinase C activation and anti-CD3 stimulation were able to induce CTLA-4 gene expression. Surprisingly, stimulation by anti-CD28 mAb alone induced CTLA-4 mRNA expression, so that signals delivered by the T cell receptor, as well as costimulatory signals would be expected to induce CTLA-4 expression. Further studies will be required to determine the mechanism of CTLA-4 gene regulation, but the ability of anti-CD28 to augment anti-CD3-induced CTLA-4 expression is reminiscent of CD28-induced effects on lymphokine synthesis (19, 20, 45), and suggest that both transcriptional and post-transcriptional mechanisms may play a role. Indeed, recent studies have shown that anti-CD2 plus anti-CD28 stimulation is capable of transcriptional and post-transcriptional augmentation of IL-2R α (CD25) mRNA expression (46).

The rabbit antiserum 1438 raised against the extracellular domain of CTLA-4 has permitted the first identification and characterization of CTLA-4 at the protein level. In experiments with biosynthetically labeled T cells, we found that CTLA-4 protein was readily detectable during the first 12 h of T cell activation. In this respect, CTLA-4 seems to differ from CD28, because we and others (29) have had difficulty detecting CD28 in primary T cells by metabolic labeling, suggesting that the turnover of CD28 protein is quite slow as compared with CTLA-4.

Using SDS-PAGE analysis, we found that CTLA-4 is expressed primarily as a 41- to 43-kDa structure on the surface of activated T cells. It is likely that CTLA-4, like CD28, is heavily glycosylated because the apparent molecular mass of CTLA-4 greatly exceeds the 23.9-kDa

structure predicted by nucleic acid sequence, and the 25-kDa product observed following in vitro translation of our CTLA-4/SV40 construct. The characteristic "signature" of CD28 in immunoprecipitations is a poorly resolved band centered at 44 kDa under reducing conditions (29–32). This is observed in lysates of leukemic cell lines as well as primary T cells. In contrast, CTLA-4 migrates as a well resolved band, and did not seem to associate with CD28 on the cell surface. Detailed studies of expression of CTLA-4 on the cell surface will have to await the development of monoclonal antibodies, although our results from immunoprecipitation experiments of surface-labeled cells have shown that small amounts of CTLA-4 are detectable on resting T cells. Further studies will be required to determine whether the small amounts of CTLA-4 expressed on resting cells is present on all $CD28^+$ T cells, or on a subset of cells such as contaminating activated T cells. The later possibility is considered unlikely because lysates from resting T cells subjected to negative selection to remove T cells expressing MHC class II antigens fails to remove material that is immunoprecipitated by 1438 (not shown).

In previous work, we suggested that CTLA-4 might exist at the cell surface in both heterodimeric as well as homodimeric forms (28). Our present results do not exclude that possibility, and indicate that CTLA-4 migrates predominantly as a monomer upon SDS-PAGE analysis, with $<10\%$ of precipitated material displaying migration expected of a disulfide-bonded dimer. CTLA-4 displayed similar migration on SDS-PAGE analysis in both reducing and nonreducing conditions, whereas immunoprecipitates of CD28 displayed characteristics of a homodimer in the same experiments. It is likely that there are intrachain disulfide bonds in CTLA-4, because the migration of CTLA-4 was slightly retarded in reducing conditions compared with nonreducing conditions (Fig. 4*B*). Preclearing experiments followed by reciprocal immununoprecipitations failed to show any association between CTLA-4 and CD28. Peptide maps of CD28 and CTLA-4 immunoprecipitates are dissimilar (data not shown), further suggesting that a CTLA-4/CD28 disulfide-linked heterodimer is not a major species on the surface of T cell blasts. However, although our studies fail to provide evidence for homo- or heterodimeric CTLA-4, it is still possible that our antiserum fails to recognize dimerized CTLA-4 and is specific for a monomeric form of CTLA-4. Similarly, although it is known that mAb 9.3 recognizes monomeric and homodimeric CD28 (29), it is possible that the mAb does not immunprecipitate heterodimeric CD28/CTLA-4 receptors. Finally, it is possible that CTLA-4 and CD28 associate by a noncovalent process independent of disulfide bond formation.

It remains to be determined why CTLA-4 should exist predominantly as a non-disulfide-bonded molecule at the cell surface. Harper and co-workers previously have noted the presence of a fifth and presumably unpaired cysteine, Cys-92, just proximal to the putatative transmembrane region (17). This residue is conserved in both human and mouse CTLA-4, as well as in CD28. In the case of CD28, this unpaired cysteine is thought be involved in the disulfide linkage that pairs CD28 as a homodimer. There is precedence for an apparently reduced cysteine at this position, however, because human CD28 has been shown to exist in both monomeric as well as dimeric forms (29). Recent evidence suggests that MHC class I proteins may exist on the surface of cells in a monomeric form as well as a heterodimeric form (47).

It is possible that CTLA-4 exists as a homodimer or as a heterodimer, but that equilibrium favors the monomer. Given the probable, but still unproven assumption that ligand binding for B7 is a property confined to dimers or other higher order multimers of CTLA-4 and CD28, then it is likely that such disulfide exchange might be a regulated process. Thioredoxin or ADF (adult T-cell leukemia-derived factor) is a protein with T cell growth properties that could be a candidate for such a process (48). Finally, even though CTLA-4, and to a lesser extent CD28, appear as monomeric structures at the cell surface under the conditions that we have explored, it is likely that signal transduction through these molecules requires clustering or aggregation, if not dimerization (49). Indeed, "spontaneous" clustering of monomeric receptor ligand pairs can occur and appears to be thermodynamically favored, as first shown by Weis and co-workers (50).

To date our studies have not revealed a functional role for CTLA-4. Since submission of our manuscript, Linsley and co-workers have published studies demonstrating that anti-CTLA-4 mAb can be synergistic with anti-CD28 mAb in their ability to augment anti-TCR-induced proliferation of preactivated T cells (51). Based on the present observations concerning the independent pattern of expression of CD28 and CTLA-4, it remains possible that instead of potential synergistic roles in T cell activation, that they may instead play distinct and independent roles. Hypothetical functions of CTLA-4 must account for the perfect conservation at the protein level of the cytoplasmic domain of CTLA-4 (17) as well as the high affinity binding of the extracellular domain of CTLA-4 for B7 (11). Predictions concerning CTLA-4 function should also take into account the similarities to CD28 in terms of structure, sequence, tissue expression, and chromosomal location.

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